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Selective interaction of JNK protein kinase isoforms with transcription factors

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The JNK protein kinase is a member of the MAP kinase group that is activated in response to dual phosphorylation on threonine and tyrosine. Ten JNK isoforms were identified in human brain by molecular cloning. These protein kinases correspond to alternatively spliced isoforms derived from the JNK1, JNK2 and JNK3 genes. The protein kinase activity of these JNK isoforms was measured using the transcription factors ATF2, Elk-1 and members of the Jun family as substrates. Treatment of cells with interleukin-1 (IL-1) caused activation of the JNK isoforms. This activation was blocked by expression of the MAP kinase phosphatase MKP-1. Comparison of the binding activity of the JNK isoforms demonstrated that the JNK proteins differ in their interaction with ATF2, Elk-1 and Jun transcription factors. Individual members of the JNK group may therefore selectively target specific transcription factors *in vivo*.

Keywords: ATF2/c-Jun/Elk-1/JNK/MAP kinase

Introduction

The AP-1 transcription factor mediates immediate-early gene expression in response to the exposure of cells to extracellular stimuli. AP-1 is composed of dimeric complexes formed by members of the Jun and Fos groups of transcription factors. AP-1 activation is caused by: (i) increased expression of Fos and Jun proteins (Vogt and Bos, 1990; Curran *et al.*, 1993); and (ii) post-translational modification of Fos and Jun by phosphorylation (Hunter and Karin, 1992). Protein kinase activities that phosphorylate and activate Jun (JNK) and Fos (FRK) have been described (Adler *et al.*, 1992a; Hibi *et al.*, 1993; Deng and Karin, 1994). Whereas the c-Fos kinase FRK is poorly understood, the c-Jun kinase JNK has been studied in detail (Davis, 1994). The JNK protein kinases phosphorylate the NH_2-terminal activation domain of c-Jun (Adler *et al.*, 1992a; Hibi *et al.*, 1993) on Ser63 and Ser73, causing increased c-Jun transcriptional activity (Pulverer *et al.*, 1991; Smeal *et al.*, 1991; Franklin *et al.*, 1992). The JNK protein kinase is markedly activated in response to treatment of cells with pro-inflammatory cytokines or exposure to environmental stress, while a lower level of JNK activation is caused by tyrosine kinase receptors (Davis, 1994). Thus, JNK can mediate the effect of extracellular stimuli on c-Jun. JNK is therefore a physiologically relevant regulator of AP-1 transcriptional activity.

The molecular cloning of human JNK (Dérijard *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994) and rat JNK (SAPK) (Kyriakis *et al.*, 1994) led to the identification of JNK as a member of the MAP kinase group of protein kinases (Davis, 1994). Activation requires the phosphorylation of JNK on Tyr and Thr within the tripeptide motif Thr–Pro–Tyr located in kinase subdomain VIII (Dérijard *et al.*, 1994). This phosphorylation is mediated by a dual specificity protein kinase. One of the JNK activators, MKK4 (also referred to as SEK1/JNKK), has been characterized (Sanchez *et al.*, 1994; Dérijard *et al.*, 1995; Lin *et al.*, 1995). In turn, MKK4 is activated by phosphorylation mediated by another protein kinase, for example MEKK1 (Minden *et al.*, 1994; Yan *et al.*, 1994). The mechanism of MEKK1 activation is not understood (Russell *et al.*, 1995), but this pathway may involve the phosphorylation of MEKK1 by the PAK protein kinase that is activated by the small GTP binding proteins Rac1 and Cdc42 (Bagrodia *et al.*, 1995; Coso *et al.*, 1995; Minden *et al.*, 1995; Olson *et al.*, 1995). The structure of the JNK signaling pathway is therefore similar to the ERK pathway that is activated by the small GTP binding protein Ras via the Raf-1 and MEK1 protein kinase cascade (Davis, 1994).

Although c-Jun is established as a JNK substrate, recent studies have led to the identification of additional targets of the JNK protein kinase. One example is the transcription factor ATF2, which is phosphorylated by JNK within the NH_2-terminal activation domain on Thr69 and Thr71 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995). Activation of the JNK signal transduction pathway causes increased phosphorylation of ATF2 on these sites *in vivo* and causes increased ATF2 transcriptional activity (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995). A second example of a target of the JNK signal transduction pathway is the Ets-domain transcription factor Elk-1 (Whitmarsh *et al.*, 1995). Serum response factor (SRF) binds with Elk-1 to the serum response element (SRE) and forms a ternary complex. Phosphorylation of the Elk-1 activation domain by JNK causes increased ternary complex formation and increased transcriptional activity (Gille *et al.*, 1995; Whitmarsh *et al.*, 1995; Zinck *et al.*, 1995). The JNK signal transduction pathway therefore activates multiple transcription factors, including ATF2, c-Jun and Elk-1.

The phosphorylation of c-Jun by JNK requires a subdomain (the δ-domain) of the NH_2-terminal region of c-Jun (Adler *et al.*, 1992a; Hibi *et al.*, 1993; Dérijard *et al.*, 1994). The δ-domain mediates a binding interaction

between c-Jun and JNK, and is physically separate from the sites of c-Jun phosphorylation by JNK (Davis, 1994). A similar JNK binding domain has been identified in the NH_2-terminal region of ATF2 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995). Deletion of the binding domain of c-Jun or ATF2 blocks the phosphorylation of these transcription factors by JNK. The binding interaction between JNK and its substrates suggests a potential mechanism for the intracellular targeting of JNK *in vivo*. This putative role for substrate binding raises questions about the specificity of JNK signaling because of the identification of multiple targets of the JNK signal transduction pathway. Two levels of complexity can be considered. First, JNK isoforms may have different binding specificities (Kallunki *et al.*, 1994; Sluss *et al.*, 1994). Second, the requirement for substrate binding by JNK may differ for individual signal transduction targets.

The purpose of these studies was to define the JNK isoforms in a single tissue by molecular cloning and to examine the specificity of the interaction of these protein kinases with signal transduction targets. We find that at least 10 JNK isoforms are expressed in human brain. Analysis of the binding activity of these JNK isoforms demonstrated that they differ in their interaction with ATF2, Elk-1 and Jun transcription factors. Furthermore, the comparison of JNK binding and phosphorylation demonstrated that some proteins (e.g. the JunB activation domain) bind JNK and are not phosphorylated, while other proteins (e.g. the Elk-1 activation domain) are phosphorylated in the absence of strong JNK binding. These data indicate that the JNK group of protein kinases consists of multiple members that may interact selectively with transcription factors *in vivo*.

Results

Molecular cloning of a group of human JNK protein kinases

Previously we have isolated one human JNK isoform (JNK1) from a fetal brain cDNA library (Dérijard *et al.*, 1994). A second human JNK isoform (JNK2) was isolated from a HeLa (Sluss *et al.*, 1994) and a Jurkat (Kallunki *et al.*, 1994) cDNA library. To examine whether these isoforms represent only part of a larger group of human JNK protein kinases, we screened adult human brain cDNA libraries for the presence of related sequences. This analysis led to the identification of 10 different JNK isoforms (Figure 1). Two of these isoforms are identical to the human JNK protein kinases described previously (Dérijard *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994). However, eight of the sequences correspond to novel JNK isoforms. A distinctive common feature of all these JNK isoforms is the conservation of the Thr–Pro–Tyr motif in protein kinase subdomain VIII (Figure 1). Previous studies have demonstrated that JNK activation is mediated by the dual phosphorylation of the Thr–Pro–Tyr sequence on Thr and Tyr (Dérijard *et al.*, 1994).

Comparison of the nucleotide sequence of the 10 JNK cDNAs demonstrates that they probably arise by alternative processing of transcripts from three genes: JNK1, JNK2 and JNK3. The JNK1 group of protein kinases includes four isoforms (Figure 1). These isoforms result from (i) the presence of alternative sequences within protein kinase subdomains IX and X; and (ii) alternative splicing that yields different COOH-termini. A similar group of four JNK2 isoforms was identified corresponding to isoforms with distinct COOH-termini and alternative sequences within protein kinase subdomains IX and X.

The third group of human JNK-related sequences (JNK3) is different from the JNK1 and JNK2 protein kinases described previously (Dérijard *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994). A major difference between JNK3 and the other groups of JNK protein kinases is the presence of an extended NH_2-terminal region fused in-frame to the conserved methionine residue that serves as the NH_2-terminus of the other JNK protein kinases (Figure 1). Two JNK3 isoforms with different COOH-termini were isolated. In contrast to observations made for JNK1 and JNK2, we did not detect JNK3 isoforms with alternative sequences within protein kinase subdomains IX and X (Figure 1).

Expression of JNK isoforms in CHO cells

The JNK protein kinase cDNAs were cloned in the expression vector pCDNA3 and expressed in Chinese hamster ovary (CHO) cells. Western blot analysis demonstrated the expression of the epitope-tagged JNK isoforms (Figure 2A). The JNK isoforms with the shorter COOH-terminal region (JNK1α1, JNK1β1, JNK2α1, JNK2β1) were detected as 46 kDa proteins. The JNK isoforms with the longer COOH-terminal region (JNK1α2, JNK1β2, JNK2α2, JNK2β2) were detected as 55 kDa proteins. Consistent with the presence of an extended NH_2-terminus in the sequence of JNK3 (Figure 1), the isoforms JNK3α1 (48 kDa) and JNK3α2 (57 kDa) were larger than the corresponding JNK1 and JNK2 proteins (Figure 2A).

Substrate phosphorylation by the JNK protein kinases

The protein kinase activity of the JNK isoforms expressed in CHO cells was measured in an immune complex kinase assay. The epitope-tagged JNK protein kinases were incubated with [γ-^{32}P]ATP and the substrates c-Jun (Dérijard *et al.*, 1994), ATF2 (Gupta *et al.*, 1995) and Elk-1 (Whitmarsh *et al.*, 1995). All of the JNK isoforms exhibited a low level of protein kinase activity. However, treatment of the cells with interleukin (IL)-1 caused increased JNK protein kinase activity (Figure 2B). Comparison of the phosphorylation of c-Jun, ATF2 and Elk-1 indicated that each of these transcription factors was phosphorylated to a similar extent by JNK.

Control experiments using JNK1α1 demonstrated that the activation caused by IL-1 was observed within 5 min and was maximal within 15 min following treatment of cells with IL-1 (Figure 3A). Dose–response analysis demonstrated that maximal JNK1α1 activation was observed in experiments using 10 ng/ml IL-1 (Figure 3B). Importantly, the ability of IL-1 to activate JNK was blocked by substitution of the Thr–Pro–Tyr dual phosphorylation motif with Ala–Pro–Phe (Figure 4). This observation is consistent with the hypothesis that JNK activation caused by IL-1 requires dual phosphorylation on Thr and Tyr. Similar observations have been made for JNK activation in cells exposed to UV radiation (Dérijard *et al.*, 1994) and tumor necrosis factor (TNF) (Sluss *et al.*, 1994).

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JNK1-α                                                    MSRSKRDNNFYSVEIGDSTFTVLKRYQN
JNK1-β                                                    ............................
JNK2-α                                                    ..D..C.SQ....QVA..........Q
JNK2-β                                                    ..D..C.SQ....QVA..........Q
JNK3-α    MSLHFLYYCSEPTLDVKIAFCQGFDKQVDVSYIAKHYN..K..V..Q.....V.............

                    I                   II                 III                IV
JNK1-α    LKPIGSGAQGIVCAAYDAILERNVAIKKLSRPFQNQTHAKRAYRELVLMKCVNHKNIIGLLNVFTP
JNK1-β    ............................................................................
JNK2-α    ..............F.TV.GIS..V.....................L.........S.......
JNK2-β    ..............F.TV.GIS..V.....................L.........S.......
JNK3-α    ..................V.D.....................................S.......

                                  V                 VIA                VIB
JNK1-α    QKSLEEFQDVYIVMELMDANLCQVIQMELDHERMSYLLYQMLCGIKHLHSAGIIHRDLKPSNIVVK
JNK1-β    ............................................................................
JNK2-α    ..T........L.............H.....................................
JNK2-β    ..T........L.............H.....................................
JNK3-α    ..T........L.....................................................

                  VII          * *  VIII              IX
JNK1-α    SDCTLKILDFGLARTAGTSFMMTPYVVTRYYRAPEVILGMGYKENVDLWSVGCIMGEMVCHKILFP
JNK1-β    ...........................................I.........IKGGV...
JNK2-α    ..............C.N...........................I.........L.KGCVI.Q
JNK2-β    ..............C.N...........................I.......A...L..V...
JNK3-α    ............................................I..........R......

                         X
JNK1-α    GRDYIDQWNKVIEQLGTPCPEFMKKLQPTVRTYVENRPKYAGYSFEKLFPDVLFPADSEHNKLKAS
JNK1-β    .T.H........................................................................
JNK2-α    .T.H..............SA..........N.........P.IK..E....WI..SE..RD.I.T.
JNK2-β    ..................SA..........N.........P.IK..E....WI..SE..RD.I.T.
JNK3-α    ........................N..........LT.P.....S..............

                            XI
JNK1-α    QARDLLSKMLVIDASKRISVDEALQHPYINVWYDPSEAEAPPPKIPDKQLDEREHTIEEWKELIYK
JNK1-β    ............................................................................
JNK2-α    ..............PD.........R....T.....A.......Q.Y.A..E....A..........
JNK2-β    ..............PD.........R....T.....A.......Q.Y.A..E....A..........
JNK3-α    ..............PA......DA.............A.V.....Q.Y....................

JNK1-α1   EVMDLEERTKNGVIRGQPSPLAQVQQ
JNK1-α2   ....................GAAVINGSQHPSSSSSVNDVSSMSTDPTLASDTDSSLEAAAGPLGCCR
JNK1-β1   ........................
JNK1-β2   ....................GAAVI.....................................
JNK2-α1   ....W...S....VKD...--..M..
JNK2-α2   ....W...S....VKD...DAG--VSSNATPSQ...-I..I.....EQ..........DAST...EG..
JNK2-β1   ....W...S....VKD...--..M..
JNK2-β2   ....W...S....VKD...DAG--VSSNATPSQ...-I..I.....EQ..........DAST...EG..
JNK3-α1   ...NS..K.....VK.....SA....
JNK3-α2   ...NS..K.....VK.....SGAAVNSSESL.P...-...I......Q............S........
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Fig. 1. Alignment of human JNK protein kinase sequences. The sequence of 10 members of the human JNK protein kinase group were compared with the PILEUP program (version 7.2; Wisconsin Genetics Computer Group). The sequences are presented in single letter code. Gaps introduced into the sequences to optimize alignments are illustrated with a dash. Residues that are identical with the sequence of JNK1α1 (or with the extended COOH-terminal region of JNK1α2) are indicated with a period. The conserved protein kinase subdomains (I–IX) are indicated (Hanks *et al.*, 1988). The sequences of JNK1α1, JNK1α2, JNK1β1, JNK1β2, JNK2α1, JNK2α2, JNK2β1, JNK2β2, JNK3α1 and JNK3α2 will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence databases under the accession nos L26318, U34822, U35004, U35005, U34821, L31951, U35002, U35003, U34820 and U34819, respectively.

Selective interaction of the JNK group of protein kinases with different transcription factors

Previous studies have established that JNK proteins bind to the substrates c-Jun (Adler *et al.*, 1992b, 1994; Hibi *et al.*, 1993; Dérijard *et al.*, 1994) and ATF2 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995). This binding interaction is mediated by a region of c-Jun and ATF2 that is separate from the sites of phosphorylation of these transcription factors by JNK. The binding specificity may be different for each JNK isoform (Kallunki *et al.*, 1994; Sluss *et al.*, 1994). We therefore compared the binding of the 10 JNK isoforms to immobilized c-Jun, ATF2 and Elk-1.

The JNK cDNAs were transcribed and translated *in vitro* in the presence of [^{35}S]methionine. The JNK proteins obtained were analyzed by SDS–PAGE and autoradiography (Figure 5A). The major protein observed corresponds to the full-length JNK protein kinase (~46 or 55 kDa). However, in the case of JNK3, a doublet of 54–57 kDa (JNK3α2) and 45–48 kDa (JNK3α1) was observed. The larger band of each doublet corresponds to the full-length JNK3 protein, while the lower band probably represents translation from the second in-frame methionine residue that is conserved with JNK1 and JNK2 (Figure 1). Equal amounts of the *in vitro* translated ^{35}S-labeled JNK isoforms were incubated for 90 min with glutathione–agarose bound to GST, GST–c-Jun, GST–ATF2 and GST–Elk-1. The agarose was washed and the bound JNK was eluted with Laemmli sample buffer, examined by SDS–PAGE, and quantitated by Phosphorimager analysis. All of the JNK isoforms bound to GST–Jun and GST–ATF2, but no binding of JNK to GST was detected (Figure 5A). Quantitative analysis of the binding assays with a

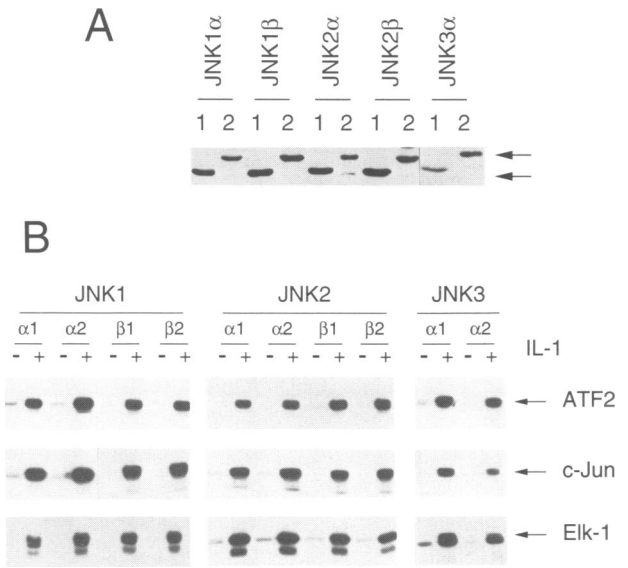

Fig. 2. Phosphorylation of ATF2, c-Jun, and Elk-1 transcription factors by JNK. Epitope-tagged JNK isoforms were expressed in CHO cells. (**A**) The level of expression of the JNK protein kinases was examined by Western blot analysis of cell lysates using the M2 monoclonal antibody. (**B**) The cells were treated with (10 ng/ml) and without IL-1 for 20 min. The JNK protein kinases were immunoprecipitated using the M2 monoclonal antibody and the JNK protein kinase activity was measured in the immune complex with [γ-^{32}P]ATP and GST–ATF2 (residues 1–109), GST–c-Jun (residues 1–79) or GST–Elk-1 (residues 307–428) as substrates.

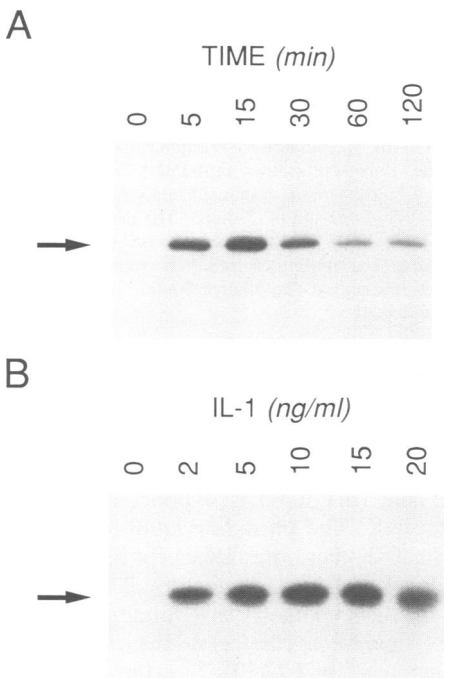

Fig. 3. Time course and dose–response of JNK activation by IL-1. Cells transfected with an expression vector for epitope-tagged JNK1α1 were treated for different times with 10 ng/ml IL-1 (**A**) or with different concentrations of IL-1 for 15 min (**B**). The JNK1α1 was immunoprecipitated with the M2 monoclonal antibody. Immune complex kinase assays were performed using the substrate c-Jun. The products of the phosphorylation reaction were resolved by SDS–PAGE and detected by autoradiography.

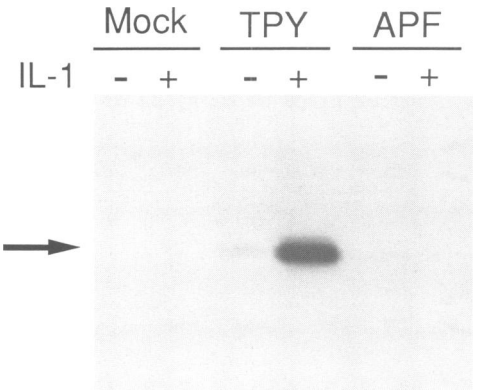

Fig. 4. The dual phosphorylation motif Thr–Pro–Tyr is required for JNK activation by IL-1. Cells were mock-transfected or transfected with expression vectors for wild-type (Thr183, Tyr185) JNK1α1, or mutated (Ala183, Phe185) JNK1α1. The epitope-tagged JNK protein kinases were immunoprecipitated with the M2 monoclonal antibody. Immune complex kinase assays were performed using the substrate c-Jun. The products of the phosphorylation reaction were resolved by SDS–PAGE and detected by autoradiography.

Phosphorimager demonstrated that the binding of JNK1β1, JNK2α1 and JNK2α2 to c-Jun was ~2-fold greater than the binding to ATF2 (Figure 5B). Furthermore, the binding of JNK2β1 and JNK2β2 to ATF2 was ~2-fold greater than the binding to c-Jun (Figure 5B). In contrast, a similar low level of binding to c-Jun and ATF2 was observed for other JNK isoforms (JNK1α1, JNK1α2, JNK1β2, JNK3α1 and JNK3α2). These data indicate that the specificity of *in vitro* transcription factor binding differs between JNK isoforms.

A subregion of the activation domain of c-Jun (Hibi *et al.*, 1993; Adler *et al.*, 1994; Dérijard *et al.*, 1994) and ATF2 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995) mediates binding to JNK and is required for the phosphorylation of these transcription factors. A primary role for the binding interaction is demonstrated by the observation that the deletion of the binding subregion of c-Jun and ATF2 abolishes the phosphorylation of these transcription factors by JNK. Since c-Jun, ATF2 and Elk-1 transcription factors are phosphorylated to a similar extent by JNK isoforms (Figure 2), we expected that the Elk-1 activation domain would also contain a JNK binding site. However, the JNK isoforms bound very weakly to the Elk-1 activation domain (Figure 5). This observation demonstrates that the binding interaction critical for the phosphorylation of c-Jun and ATF2 is not required for the *in vitro* phosphorylation of the Elk-1 activation domain by JNK.

It is unclear whether the Elk-1 activation domain is an exception to a general requirement for JNK binding for substrate phosphorylation or whether the role of the binding interaction is relevant specifically only to the transcription factors c-Jun and ATF2. To address this question, we have searched for additional JNK substrates. This analysis has demonstrated that JNK exhibits a high level of substrate specificity (Davis, 1994). Consequently, most proteins we have tested are not phosphorylated by JNK. However, members of the Jun family were phosphorylated by JNK (Figure 6). We compared the phosphorylation of c-Jun with JunB and JunD in an immune complex kinase assay using epitope-tagged

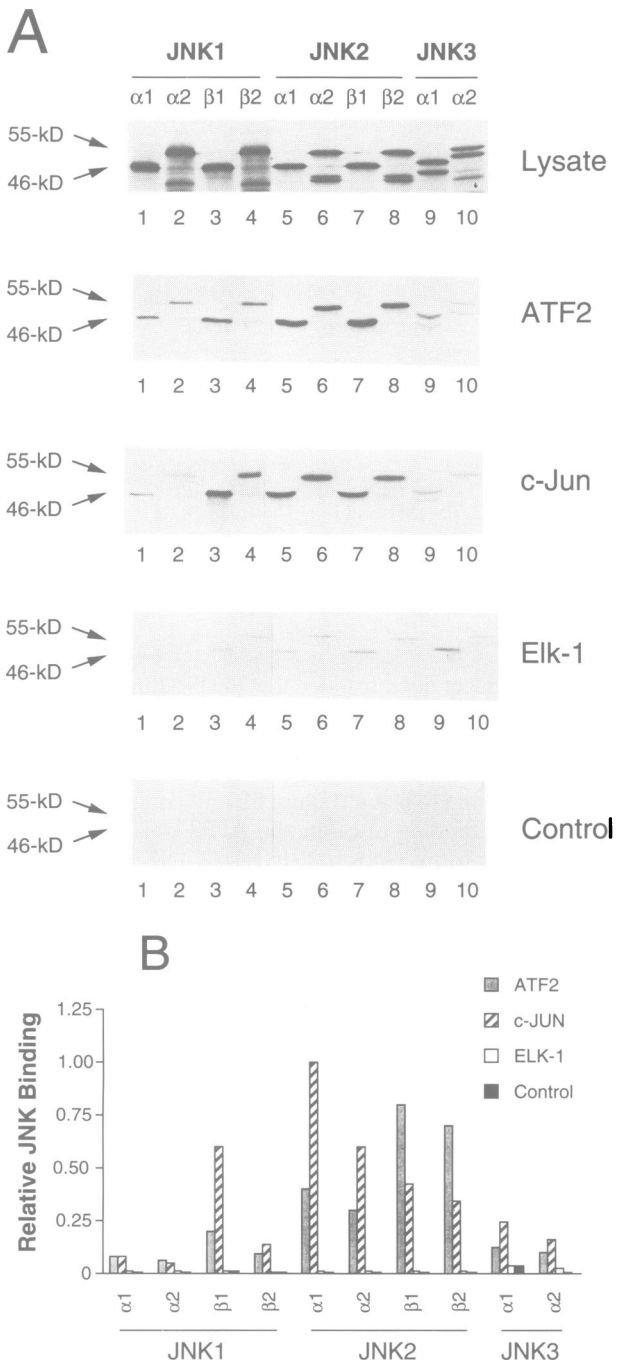

Fig. 5. Interaction of JNK with ATF2, c-Jun and Elk-1 transcription factors. The JNK isoforms were expressed by *in vitro* translation with [^{35}S]methionine. (**A**) The translated JNK protein kinases were examined by SDS–PAGE and autoradiography (Lysate). Each JNK isoform was incubated with immobilized GST, GST–ATF2 (residues 1–109), GST–c-Jun (residues 1–79) and GST–Elk-1 (residues 307–428). The protein complexes were washed and the bound JNK was detected by SDS–PAGE and autoradiography. (**B**) The binding of each JNK isoform was quantitated by PhosphorImager analysis (Molecular Dynamics Inc.). The binding of the JNK isoforms is presented relative to the JNK2α1 interaction with c-Jun. The data presented are the average of results obtained from two independent experiments.

JNK2α2. This analysis demonstrated that JunB is not a JNK substrate and that JunD is a weak JNK substrate compared with c-Jun (Figure 6A). Analysis of the binding interaction revealed that JunD bound weakly to JNK2α2

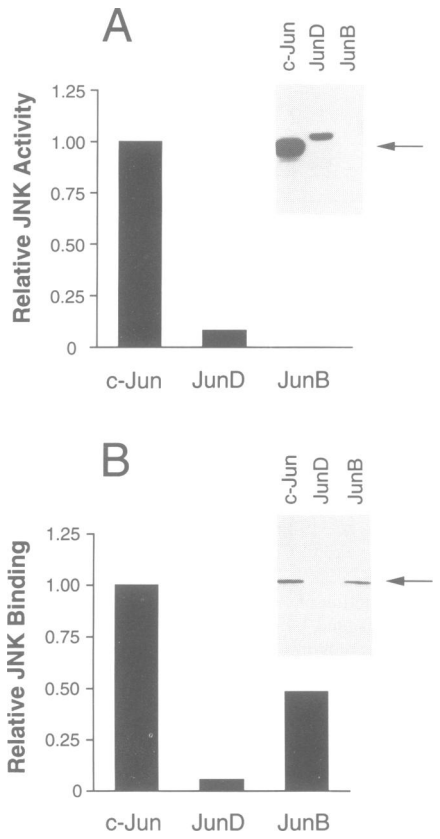

Fig. 6. JNK interaction with different members of the Jun group of transcription factors. (**A**) Epitope-tagged JNK2α2 was expressed in CHO cells. The cells were treated with IL-1 (10 ng/ml) for 20 min. The JNK protein kinases were immunoprecipitated using the M2 monoclonal antibody and the JNK protein kinase activity was measured in the immune complex with [γ-^{32}P]ATP and Jun transcription factors c-Jun (residues 1–79), JunB (residues 1–100) and JunD (residues 1–115). The phosphorylated Jun proteins were resolved by SDS–PAGE and quantitated by PhosphorImager (Molecular Dynamics Inc.) analysis (arbitrary units). (**B**) JNK2α2 translated *in vitro* with [^{35}S]methionine was incubated with immobilized GST–c-Jun, GST–JunB and GST–JunD. The protein complexes were washed and the bound JNK was detected by SDS–PAGE and autoradiography. The binding of each JNK isoform was quantitated by PhosphorImager analysis. The relative binding of JNK2α2 to the Jun proteins is presented.

(Figure 6B). In contrast, the binding of JNK2α2 to JunB was 2-fold less than the binding c-Jun. Together, these data demonstrate that (i) c-Jun is an excellent substrate that binds JNK; (ii) JunB binds to JNK, but is not a substrate; and (iii) JunD is a poor substrate that binds weakly to JNK. The phosphorylation and binding interaction with JNK therefore differs for each member of the Jun family of transcription factors. This finding is consistent with the hypothesis that binding and phosphorylation are separate attributes of the *in vitro* interaction of proteins (e.g. ATF2, Elk-1, c-Jun, JunB and JunD) with JNK.

Inactivation of JNK protein kinase isoforms by MAP kinase phosphatase

The ERKs have been demonstrated to be inactivated by a group of dual specificity phosphatases that include MKP-1 (CL-100 and 3CH134), MKP2, B23 and PAC-1 (Alessi *et al.*, 1993; Sun *et al.*, 1993; Zheng and Guan,

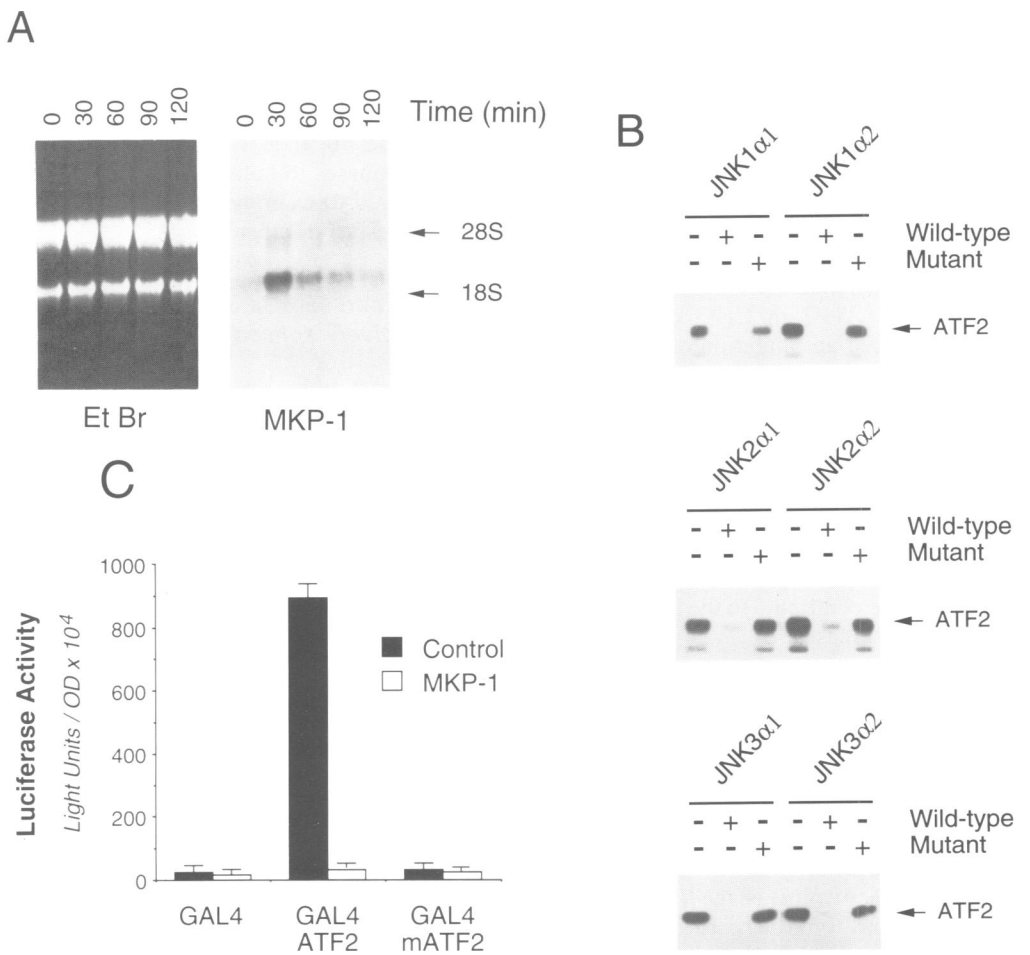

Fig. 7. Inactivation of JNK by the MAP kinase phosphatase MKP-1. (**A**) MKP-1 expression was examined by Northern blot analysis of RNA isolated from IL-1-treated CHO cells. A photograph of the ethidium bromide (EtBr)-stained gel showing the relative loading of RNA in each lane and an autoradiograph of the blot hybridized to an MKP-1 probe is presented. The position of the 28S and 18S rRNA is illustrated. (**B**) CHO cells were transfected with a JNK expression vector together with the empty expression vector pSG5 (Control) or a vector encoding wild-type MKP-1, or a catalyically inactive mutant (Cys258 replaced by Ser). The JNK protein kinases were isolated by immunoprecipitation using the M2 monoclonal antibody and the JNK protein kinase activity was detected in the immune complex using [γ-^{32}P]ATP and the transcription factor ATF2 as substrates. (**C**) The transcriptional activity of wild-type ATF2 (Thr69, Thr71) and mutated ATF2 (Ala69, Ala71) (mATF2) was measured using a luciferase reporter gene. The effect of the pSG5 vector (Control) or the MKP-1 expression vector on the reporter gene expression is presented. Transfection efficiency was monitored with a β-galactosidase expression vector. The data are presented as the ratio of luciferase activity (light units) to β-galactosidase (OD units) measured in the cell extracts.

1993; Ishibashi *et al.*, 1994; Ward *et al.*, 1994; Misra-Press *et al.*, 1995). However, the role of these enzymes in the regulation of JNK is unclear because evidence has been reported indicating that MAP kinase phosphatase may not inactivate JNK (Sun *et al.*, 1994). The failure of MAP kinase phosphatase to inactivate JNK1α1 (Sun *et al.*, 1994) may reflect a specificity of these phosphatases for the ERK group of MAP kinases. Alternatively, JNK inactivation by MAP kinase phosphatase may differ between JNK isoforms (Liu *et al.*, 1995). We therefore examined the potential regulation of JNK isoforms by MAP kinase phosphatase.

In initial experiments, we observed that bacterially expressed GST–PAC1, but not a catalytically inactive mutant GST–PAC1 (Cys257 replaced by Ala), inactivates JNK1α1 protein kinase activity *in vitro* (data not shown). Furthermore, co-expression of wild-type PAC1, but not the catalytically inactive mutant PAC1, together with JNK1α1 blocked UV-stimulated JNK protein kinase activity (data not shown). These data suggest that PAC-1 may function as a JNK phosphatase *in vivo*. However, as PAC1 is expressed exclusively in T cells (Rohan *et al.*, 1993), it is unclear whether PAC1 is representative of other MAP kinase phosphatases. We therefore examined the possible role of MKP-1, a MAP kinase phosphatase that is expressed in many tissues (Charles *et al.*, 1992; Keyse and Emslie, 1992).

The expression of MKP-1 in CHO cells was examined by Northern blot analysis. A low level of MKP-1 was detected in serum-starved CHO cells (Figure 7A). Treatment with IL-1 caused a rapid increase in MKP-1 expression (Figure 7A). Previous studies have demonstrated increased MAP kinase phosphatase expression following ERK activation (Sun *et al.*, 1993; Ward *et al.*, 1994). However, IL-1 treatment of CHO cells causes JNK activation without changes in ERK protein kinase activity (Whitmarsh *et al.*, 1995). Thus, MKP-1 expression is not tightly linked to the regulation of ERKs. MKP-1 expression may therefore be relevant to the JNK protein kinase signal transduction pathway.

To examine whether MKP-1 inactivates JNK, we expressed wild-type MKP-1 and a catalytically inactive MKP-1 mutant (Cys258 replaced by Ser) in CHO cells. We observed that MKP-1, but not the catalytically inactive mutant, inhibited IL-1-stimulated JNK protein kinase activity in experiments using JNK1, JNK2 and JNK3 isoforms (Figure 7B). The inhibitory effect of MKP-1 on JNK activation suggests that MKP-1 may be a physiological regulator of the JNK protein kinase signal transduction pathway. To test this hypothesis, we examined the transcriptional activity of ATF2 in CHO cells. It was observed that MKP-1 caused an inhibition of ATF2 transcriptional activity measured with a luciferase reporter gene (Figure 7C). The ability of MKP-1 to inhibit ATF2 transcriptional activity was similar to the effect of mutation of the sites of ATF2 phosphorylation by JNK, Thr69 and Thr71 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995). These data demonstrate that MKP-1 can function as an inhibitor of the JNK protein kinase signal transduction pathway (Figure 7). MAP kinase phosphatases may therefore contribute to the physiological regulation of JNK protein kinases.

Discussion

Regulation of JNK protein kinase activity by inflammatory cytokines and environmental stress

Treatment of cells with inflammatory cytokines or exposure to environmental stress causes activation of the JNK protein kinase signal transduction pathway (Davis, 1994). The mechanism of JNK activation is mediated by dual phosphorylation on Thr and Tyr (Dérijard *et al.*, 1994). Interestingly, the activators of the JNK signaling pathway [e.g. environmental stress (Keyse and Emslie, 1992) and the cytokine IL-1 (Figure 7)] also cause increased expression of the MKP-1 dual specificity MAP kinase phosphatase. A role for MAP kinase phosphatase in the regulation of p38 MAP kinase (Raingeaud *et al.*, 1995) and ERK (Sun *et al.*, 1993; Ward *et al.*, 1994) has been proposed. In contrast, the relevance of MKP-1 to the JNK signal transduction pathway is unclear (Sun *et al.*, 1994; Liu *et al.*, 1995). However, we found that MKP-1 expression blocks activation of the JNK signal transduction pathway (Figure 7). The demonstration that multiple JNK isoforms are inactivated by MKP-1 (Figure 7) indicates that MKP-1 expression in stressed or cytokine-treated cells may contribute to the regulation of the JNK signal transduction pathway.

The human JNK protein kinase group includes multiple isoforms

Extensive screening of human adult brain cDNA libraries has led to the isolation of 10 JNK isoforms (Figure 1). The identification of multiple JNK isoforms in adult brain (Figure 1) contrasts with previous studies in which only a single JNK isoform (JNK1α1) was identified in fetal brain (Dérijard *et al.*, 1994). Northern blot analysis demonstrates both increased complexity and an increased level of JNK expression in adult brain compared with fetal brain (Dérijard *et al.*, 1994), indicating that JNK expression is developmentally regulated in the brain.

Analysis of the nucleotide sequence of the 10 JNK isoforms identified in human adult brain indicates that they arise from the alternative processing of transcripts from three different genes (JNK1, JNK2 and JNK3). Our studies do not exclude the possibility that additional JNK isoforms are expressed in the brain. However, it is likely that these 10 JNK isoforms represent the major JNK protein kinases in human adult brain. Further studies will be required to examine whether additional JNK isoforms are expressed in other tissues.

46 kDa and 55 kDa JNK isoforms correspond to alternatively spliced variants of JNK1, JNK2 and JNK3

Analysis of JNK protein kinases using an in-gel protein kinase assay have demonstrated the electrophoretic separation of two classes of JNK with apparent M_rs of 46 and 55 kDa (Hibi *et al.*, 1993). It has been proposed previously that JNK1 and JNK2 correspond to the 46 and 55 kDa isoforms, respectively (Dérijard *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994). However, the results of the present study indicate that the 46 and 55 kDa isoforms are encoded by both JNK1 and JNK2 genes. Alternative processing of RNA transcripts results in the deletion or retention of five nucleotides in the coding region for the JNK COOH-terminus (Figure 1). Deletion of the five nucleotides changes the reading frame causing the fusion of an extended COOH-terminal region on the 46 kDa JNK protein kinase to create the 55 kDa JNK protein kinase. This alternative processing was detected for transcripts of the JNK1, JNK2 and JNK3 genes (Figure 1). Thus, the JNK isoforms with approximate M_rs of 46 and 55 kDa detected by in-gel kinase assays represent a mixture of JNK isoforms. In-gel kinase assays, therefore, do not distinguish adequately between JNK protein kinase isoforms.

JNK binds to the transcription factors ATF2 and c-Jun

Translation of JNK *in vitro* yielded several [^{35}S]methionine-labeled fragments in addition to the expected full-length JNK protein kinases (Figure 5). Significantly, JNK binding to ATF2 and c-Jun was only detected for the full-length forms of JNK1 and JNK2 (46 and 55 kDa). No binding of the JNK fragments obtained by *in vitro* translation was observed (Figure 5). The failure of the JNK fragments to bind ATF2 and c-Jun provides an internal control for the binding specificity of the full-length JNK protein kinases. These data establish further that a short region of JNK, by itself, is insufficient for the observed binding interaction. It is possible that sequences critical for the binding interaction are located in both the NH_2- and COOH-terminus of JNK. However, as JNK protein kinases are predicted to have a compact globular structure (Kallunki *et al.*, 1994), it is likely that the JNK fragments obtained by *in vitro* translation are misfolded. The failure of the JNK fragments to bind ATF2 and c-Jun may therefore reflect a requirement for correct protein folding to create the surface of the JNK protein kinase that interacts with these transcription factors. Further studies designed to examine the physical structure of the JNK–transcription factor complex are necessary to test this hypothesis.

```
c-Jun       MTAKMETTFY-DDAL--------------------NASFLQSESGAYGTSNPKILKQSMT
JunD            ...P..GEE..SGLAAGASSVAGATGAPGGGGF.PPGRA-FPGAPPTSSMLK.DAL.
JunB        .CT...QP..H..SY--------------------A.AGYGRSP.SLSLHDY.L..PTLA
Consensus       ME  FY   S                      A                  K

                                          *         *
c-Jun       LNLADP-VGSLK-PHLRAKNS--------DLLTSPDVGL-LKLASPELERLIIQSSNGHIT
JunD        .S..EQGAAG..PGSAT.PSALRPDGAPDG..A...L..-.............-...LV.
JunB        ......-YRG..G.GA.GPGPEGSGAGSYFSGQGS.T.AS.....T......VPN...V..
Consensus   L LA      LK                        D G  LKLAS ELERLI   SNG  T
```

Fig. 8. Comparison of the NH_2-terminal regions of the Jun group of transcription factors. The sequence of the NH_2-terminal regions of c-Jun (residues 1–79), JunD (residues 1–115) and JunB (residues 1–100) were compared with the PILEUP program (version 7.2; Wisconsin Genetics Computer Group). The sequences are presented in single letter code. Gaps introduced into the sequences to optimize alignments are illustrated with a dash. Identical residues are indicated with a period. The δ-domain of c-Jun is overlined. The c-Jun phosphorylation sites (Ser–Pro) are indicated with an asterisk in bold (Ser63 and Ser73).

Alternative exons within the protein kinase domain contribute to the binding selectivity of JNK

In vitro binding studies demonstrate that the full-length JNK protein kinase sequence is required for the binding interaction between JNK and transcription factors (Figure 5). However, the specificity of this binding interaction differs between JNK isoforms. Comparison of the binding of JNK2 isoforms with c-Jun and ATF2 indicates that the level of JNK2α1 and JNK2α2 binding to c-Jun is ~2-fold greater than the binding to ATF2 (Figure 5). In contrast, the binding of JNK2β1 and JNK2β2 to ATF2 is ~2-fold greater than the binding to c-Jun (Figure 5). The difference in the structure of the JNK2α and JNK2β isoforms is a change in the sequence located between kinase subdomains IX and X (Figure 1). Together, these data point to a role for the sequence within subdomains IX and X of the JNK protein kinase in determining binding selectivity (Figure 5). This is consistent with the results of previous studies of JNK chimeras that have established an important role for subdomains IX and X in the high affinity binding of JNK to c-Jun (Kallunki *et al.*, 1994). Further evidence in favor of this hypothesis was obtained from the finding that JNK1β1 [which is homologous to JNK2α within subdomains IX and X (Figure 1)] binding to c-Jun is greater than the binding detected for JNK1α and JNK3α isoforms (Figure 5).

Comparison of the nucleotide sequence of α and β isoforms of JNK1 and JNK2 indicates that they differ only in subregions IX and X, suggesting that these protein kinases arise by alternative splicing of transcripts of the JNK1 and JNK2 genes. Similar processing of JNK3 transcripts is also possible; however, alternative α and β isoforms of JNK3 were not identified in our screens of human brain cDNA libraries (Figure 1). Confirmation that the α and β JNK isoforms arise by alternative splicing was obtained by examination of JNK2 genomic clones. Sequence analysis demonstrated that the alternative α and β isoforms are encoded by two exons that are separated by an intron of ~300 bp (data not shown). Further studies of the JNK2 genomic clones by fluorescence *in situ* hybridization of normal human metaphase chromosomes demonstrated that the JNK2 gene locus is 5q35 (data not shown).

Interaction of JNK protein kinases with members of the Jun group of transcription factors

The Jun family of transcription factors includes c-Jun, JunB and JunD (Vogt and Bos, 1990). These proteins share similar sequences in their NH_2-terminal activation domains and COOH-terminal DNA binding domains. However, the properties of these transcription factors are distinct. For example, while c-Jun, JunB and JunD can function as transcriptional activators (Hirai *et al.*, 1989, 1990; Schütte *et al.*, 1989b), JunB inhibits the transcriptional activation caused by c-Jun (Chiu *et al.*, 1989; Schütte *et al.*, 1989b; Deng and Karin, 1993). Furthermore, the ability of c-Jun to transform fibroblasts (Schütte *et al.*, 1989a) contrasts with the abilities of JunB and JunD to inhibit fibroblast transformation (Schütte *et al.*, 1989b; Pfarr *et al.*, 1994). It is established that JNK causes phosphorylation of c-Jun (Davis, 1994). However, the role of JNK in the regulation of JunB and JunD is unclear (Franklin *et al.*, 1992; Deng and Karin, 1993). The results of this study demonstrate that JunB is not a JNK substrate and that JunD is a poor substrate for JNK phosphorylation compared with c-Jun (Figure 6).

Studies of c-Jun phosphorylation demonstrate that the binding of JNK to the δ subregion of the NH_2-terminal activation domain of c-Jun is required for the phosphorylation of c-Jun on Ser63 and Ser73 (Adler *et al.*, 1992a,b, 1994; Hibi *et al.*, 1993; Davis, 1994; Dérijard *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994). The δ subregion of c-Jun is poorly conserved in the NH_2-terminal regions of JunB and JunD (Figure 8). Analysis of the JNK interaction with Jun family proteins demonstrated that JNK bound poorly to JunD compared with c-Jun. In contrast, JNK binding to JunB was 2-fold less than the binding to c-Jun (Figure 6). The low level of JNK binding to JunD probably accounts for the low level of JunD phosphorylation caused by JNK (Figure 6). However, the binding of JunB to JNK is anomalous because JNK does not phosphorylate JunB. It is likely that the absence of JunB phosphorylation is caused by the lack of conservation of the Ser–Pro JNK phosphorylation sites that are present in the c-Jun activation domain (Figure 8). In contrast, these phosphorylation sites are conserved in JunD (Figure 8).

Function of the binding interaction between JNK and substrates

The analysis of JNK interaction with Jun family proteins demonstrates that c-Jun, JunB and JunD are differentially regulated by JNK. Thus, c-Jun binds and is phosphorylated by JNK; JunB binds to JNK and is not a JNK substrate; while JunD is a poor JNK substrate that binds weakly to JNK (Figure 6). The binding and kinase assays were not performed under identical conditions (Figure 6). However, the lack of correlation between the observed binding and

phosphorylation indicates that these are separate attributes of the JNK interaction with Jun family proteins. This analysis raises questions about the physiological function of the JNK binding interaction. In the case of c-Jun (Hibi *et al.*, 1993; Adler *et al.*, 1994; Dérijard *et al.*, 1994) and ATF2 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995), the binding interaction is required for substrate phosphorylation by JNK (Davis, 1994). However, the role of JNK binding differs for other proteins. Thus, the Ets-domain transcription factor Elk-1 is an excellent JNK substrate (Whitmarsh *et al.*, 1995) in the absence of detectable binding (Figures 2 and 5). In contrast, JunB binds to JNK and is not a substrate (Figure 6). Together, these observations indicate that JNK binding may serve multiple roles. (i) JNK binding is required for the phosphorylation of some transcription factors (e.g. c-Jun and ATF2), but not others (e.g. Elk-1). (ii) JNK binding may serve a general role in the targeting of JNK *in vivo*. For example, the presence of JNK in AP-1 complexes may lead to the phosphorylation of AP1-associated proteins, such as NFAT. (iii) JNK binding may regulate protein function directly. For example, the binding of JNK to the NH_2-terminus of JunB may contribute to the low transcriptional activity of this Jun family member by sequestering the activation domain.

Conclusions

The JNK protein kinases are activated by treatment of cells with inflammatory cytokines and by exposure to environmental stress. This group of protein kinases includes at least 10 members that interact selectively with ATF2, Jun and Elk-1 transcription factors. The expression of multiple JNK isoforms provides a mechanism for the generation of tissue-specific responses to the activation of the JNK signal transduction pathway.

Materials and methods

Molecular cloning and plasmid construction

The human JNK isoforms cloned from human fetal brain (JNK1α1) and HeLa (JNK2α2) cDNA libraries have been described (Dérijard *et al.*, 1994; Sluss *et al.*, 1994). Human JNK2α2 has also been isolated from a Jurkat cDNA library (Kallunki *et al.*, 1994). Eight novel JNK isoforms were isolated from human adult brain cDNA libraries using methods that have been described previously in detail (Sluss *et al.*, 1994). The Flag epitope (-Asp-Tyr-Lys-Asp-Asp-Asp-Aps-Lys-; Immunex Corp.) was inserted between codons 1 and 2 of the protein kinases by insertional overlapping PCR (Ho *et al.*, 1989). The epitope-tagged cDNAs were cloned into the mammalian expression vector pCDNA3 (Invitrogen Inc.) using the *Hin*dIII–*Bam*HI restriction sites for JNK1/2 and the *Hin*dIII–*Xba*I restriction sites for JNK3. The JNK1, JNK2 and JNK3 cDNAs were also cloned in the expression vector pCMV5 (Andersson *et al.*, 1989) using the *Hin*dIII and *Bam*HI restriction sites. The mutated (Ala183, Tyr185) JNK1 expression vector has been described (Dérijard *et al.*, 1994). The sequence of all constructs was confirmed by automated sequencing using a model 373A machine (Applied Biosystems Inc.). The vector pSG5 (Stratagene Inc.) that expresses wild-type mouse MKP-1 (3CH134) and the catalytically inactive mutant (Cys258 replaced by Ser) was provided by N.Tonks (Sun *et al.*, 1993). The wild-type PAC1 and catalytically inactive (Cys257 replaced by Ser) expression vectors have been described previously (Ward *et al.*, 1994). The human MKP-1 (CL100) expression vector (pMT-CL100) was provided by K.Kelly (Keyse and Emslie, 1992).

Preparation of recombinant proteins

Bacterial expression of GST–Jun (residues 1–79 and 1–223) (Dérijard *et al.*, 1994), GST–ATF2 (residues 1–109) (Gupta *et al.*, 1995) and GST–Elk-1 (residues 307–428) (Whitmarsh *et al.*, 1995) and GST–PAC1 (wild-type and mutated, Cys257 replaced by Ser) (Ward *et al.*, 1994) have been described. The GST–JunB and GST–JunD expression plasmids were constructed using the vector pGEX-5X1 (Pharmacia-LKB Biotechnology Inc.) and PCR fragments corresponding to JunB (residues 1–100) and JunD (residues 1–115). The structure of the bacterial expression vectors was confirmed by automated sequencing using a model 373A machine (Applied Biosystems Inc.). The GST fusion proteins were purified by glutathione affinity chromatography (Smith and Johnson, 1988).

Tissue culture

CHO cells were maintained in Ham's F12 medium supplemented with fetal calf serum (5%) (Gibco-BRL). The cells were transfected with the lipofectamine reagent according to the manufacturer's recommendations (Gibco-BRL) and treated with murine IL-1 (Genzyme Corp.).

Protein kinase assays

The cells were solubilized with Triton lysis buffer [TLB: 20 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM Na orthovanadate, 2 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin] following treatment with 10 ng/ml IL-1. The extracts were centrifuged at 14 000 *g* for 15 min at 4°C. Immune complex kinase assays were performed using extracts prepared from cells expressing epitope-tagged JNK. The epitope-tagged protein kinases were immunoprecipitated by incubation for 1 h at 4°C with the M2 Flag monoclonal antibody (IBI-Kodak) bound to protein G–Sepharose (Pharmacia-LKB Biotechnology Inc.). The immunoprecipitates were washed twice with TLB and twice with kinase buffer [20 mM HEPES (pH 7.4), 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM Na orthovanadate]. The kinase assays were initiated by the addition of 1 μg of substrate proteins and 50 μM [γ-^{32}P]ATP (10 Ci/mmol) in a final volume of 22 μl. The reactions were terminated after 15 min at 30°C by addition of Laemmli sample buffer. Control experiments demonstrated that the phosphorylation reaction was linear with time for at least 30 min under these conditions. The phosphorylation of the substrate proteins was examined by SDS–PAGE followed by autoradiography.

Western blot analysis

The cells were solubilized with TLB and centrifuged at 14 000 *g* for 15 min at 4°C. JNK expression in the cell extracts was detected by immunoblot analysis using the M2 monoclonal antibody (IBI-Kodak). Immune complexes were visualized using enhanced chemiluminescence detection (Amersham International PLC).

Northern blot analysis

CHO cells in 100 mm dishes were serum starved for 18 h. The cells were treated with 10 ng/ml IL-1 for different times. Total RNA was isolated using RNA-STAT-60™ following the manufacturer's recommendations (Tel-Test-B Inc). The RNA was fractionated on a formaldehyde–agarose gel, transferred to a Duralon membrane (Stratagene Inc.), and hybridized with an MKP-1 probe at 42°C. The probe was a 1950 bp *Eco*RI fragment of the MKP1 cDNA isolated from the plasmid pMT-CL100 and was labeled by random priming (Boehringer Mannheim) with [α-^{32}P]ATP (Amersham International). The blot was washed with 2× SSC, 0.1% SDS for 20 min at 25°C and twice with 0.1× SSC, 0.1% SDS at 50°C for 15 min. The MKP-1 mRNA hybridized to the probe was detected by autoradiography.

Binding assays

The JNK cDNAs cloned in the vector pCDNA3 (Invitrogen Inc.) were transcribed and translated *in vitro* using the TNT®-coupled reticulocyte lysate system (Promega Biotec Inc.). T7 RNA polymerase, 1 μg of plasmid DNA and 40 μCi [^{35}S]methionine (Amersham International PLC) was incubated with reticulocyte lysate for 90 min at 30°C in a final volume of 50 μl. Equal amounts of ^{35}S-labeled JNK proteins were incubated with GST fusion proteins immobilized on glutathione–agarose beads (Pharmacia-LKB Biotechnology Inc.) in 500 μl of TLB. Equal amounts of each GST fusion protein (5 μg) were employed. The beads were incubated for 90 min at 4°C on an end-over-end mixer and then washed four times with TLB. The bound JNK was eluted from the glutathione beads with Laemmli sample buffer, analyzed by SDS–PAGE and detected by autoradiography. The bound JNK was quantitated by PhosphorImager analysis (Molecular Dynamics Inc.).

Reporter gene expression

The activity of GAL4–ATF2 was measured in co-transfection assays with the reporter plasmid pG5E1bLuc (Gupta *et al.*, 1995). This reporter

plasmid contains five GAL4 sites cloned upstream of a minimal promoter element and the firefly luciferase gene (Seth *et al.*, 1992). Transfection efficiency was monitored using a control plasmid that expresses β-galactosidase (pCH110; Pharmacia-LKB Biotechnology Inc.). The luciferase and β-galactosidase activities in cell extracts were measured (Gupta *et al.*, 1993).

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Note added in proof

The sequence of human JNK3α1 was reported by A.A.Mohit, J.H.Martin and C.A.Miller (1995) *Neuron*, **14**, 67–78. The conclusion that MKP-1 expression correlates with stress signals rather than ERK activation has been reported by D.Bokemeyer, A.Sorokin, M.Yan, N.G.Ahn, D.J.Templeton and M.J.Dunn (1996) *J. Biol. Chem.*, **271**, 639–642.